Pak. J. Bot., 42(5): 3173-3181, 2010.

ASSESSMENT OF GENETIC DIVERSITY AMONG THE VARIETIES OF *GOSSYPIUM ARBOREUM* AND *GOSSYPIUM HIRSUTUM* THROUGH RANDOM AMPLIFICATION OF POLYMORPHIC DNA (RAPD) AND SIMPLE SEQUENCE REPEAT (SSR) MARKERS

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Abstract

The relative efficiency of RAPD and SSR markers was assessed for their suitability and reliability for estimating genetic diversity in cotton. Different varieties of cotton belonging to *Gossypium arboreum* and *Gossypium hirsutum* were used by manipulating a number of different loci as marker estimates. Twenty one primers were used. The cotton varieties appeared highly polymorphic and the mean polymorphism information content (PIC) values were 0.656 (RAPD) and 0.290 (SSR) while the average genetic distances were 0.371(RAPD) and 0.320 (SSR) in case of *Gossypium hirsutum*. In case of *Gossypium arboreum* the mean polymorphism information content (PIC) values were 0.578 (RAPD) and 0.183 (SSR) and the average genetic distances were 0.371 (RAPD) and 0.410 (SSR). Of the 21 primers used for the diversity study of the *Gossypium hirsutum* 14 (66.66%) RAPD and 04 (19.04%) SSR primers were highly informative while 13 (61.90%) RAPD and 02 (09.52%) SSR primers proved to be highly informative in case of *Gossypium arboreum*.

Introduction

Cotton is cultivated on a large area in the world primarily as a source of fiber and occupies a pivotal position in the world economy. In Pakistan, cotton is an important cash crop and the most significant source of foreign exchange earnings. The genus *Gossypium* contains 50 species (Poehlman & Sleper, 1995). Four species are cultivated, including two tetraploid *G. hirsutum* and *G. barbadense* (2n = 4x = 52), and two diploid *G. arboreum* and *G. herbaceum* (2n = 2x = 26). The existence of genetic diversity in an ecosystem or genepools ensures the adaptation of species to environmental changes and thus its survival. The existence of genetic variability is a prerequisite for the evolution of superior cotton varieties through selection and hybridization. The variety specific DNA markers in a cotton breeding program for variety registration, plant patents, confirmation of the parentage of hybrids, breeder's right protection and early detection of agronomic and economic traits as an aid to marker assisted selection are very much needed and are widely employed (Brubaker & Wendel, 1994; Asif *et al*., 2009). Therefore, it is imperative to locate and utilize the diversity present in cotton. Various methods are in practice to locate and quantify the extent of existing genetic diversity but the DNA markers such as RAPD, RFLP, AFLP, SSR and EST-SSR are among the most reliable and sophisticated methods (Ahmad *et al*., 2007).

Polymerase chain reaction (PCR) technology has promoted the development of a range of molecular assay systems, which could spot polymorphism at the DNA level. The past restrictions associated with pedigree data, morphological, physiological and cytological markers for assessing genetic diversity in cultivated and wild plant species have largely been circumvented by the development of DNA markers such as Randomly Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeat (SSR).

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The present study aims to assess the relative effectiveness of RAPD and SSR markers for diversity analysis among the varieties belonging to G. *arboreum* (diploid) and *G. hirsutum* (tetraploid) species.

Materials and Methods

Plant material and DNA extraction: Twenty five varieties, 19 belonging to *G. hirsutum* (FH-87, CIM-240, MNH-554, NIAB-78, FH-2006, MNH-642, CIM-200, NIAB-884, CIM-243, FH-628, MNH-149, FH-634, NIBGE-2, FH-901, 138-F, CIM-70, ALA, FS-628 and AFGHANISTAN) and six belonging to *G. arboreum* (FDH-786, FDH-306, FDH-228, FDH-170, FDH-300 and FDH-113) were selected and planted in pots. DNA was extracted from the tender leaves of three week old seedlings using CTAB method (Doyle & Doyle, 1990).

Molecular analysis

RAPD analysis: The genomic DNA was subjected to polymerase chain reaction. Seventy eight random decamer primers (Gene Link, New York, USA) were screened to identify those giving good and scorable amplification products. Twenty one oligonucleotide primers were finally selected for RAPD analysis. Each reaction mixture (25 µl) for PCR amplification consisted of 1X reaction buffer (10 mM Tris-HCl, pH 8.3 and 50 mM KCl), 3.0 mM $MgCl_2$, 2U Taq DNA polymerase; 200 mM each dATP, dTTP, dCTP and dGTP (all reagents from Fermentas), 0.5 mM decamer primer (Gene Link, New York, USA) and 40 ng genomic DNA template. The temperature profile for the reactions is given as: Hot start at 95°C (only at the start of reaction) for 5 min., denaturation at 95°C for 1min., primer annealing at 34°C for 1 min., extension at 72°C for 2 min. and final extension at 72°C (only at the end of reaction) for 10 min. The amplified products were separated by electrophoresis and polymorphism was detected on 1.2% agarose gel by using ethidium bromide.

SSR analysis: A set of 21 SSR primer pairs (JESPR Series, Invitrogen Life Technologies) (Reddy *et al*., 2001) were used for amplification in the same varieties. PCR reaction (20 µl) mixture contained DNA, 15 ng/µl; 10X buffer; 10 mM dNTPs; 50 mM $MgCl_2$; 10 µM each of forward and reverse primers. The amplification was carried out in thermal cycler (Eppendorf AG No. 533300839, Germany) using a touch-down program configured having a denaturation step of 5 min at 94°C followed by 10 cycles of 30 s at 94°C, 30 s at 65°C decreasing by 1°C per cycle until 55°C, and 1 min at 72°C. Then 30 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C. The program ended with one final extension at 72°C for 10 min. The amplified products were separated by electrophoresis and polymorphism was detected on 3% agarose gel by using Ethidium bromide.

Data analysis: The electrophoresed gels were examined under ultra violet Transilluminator and photographed using SyneGene Gel Documentation System. All amplification products were scored as present (1) or absent (0) for each of the 25 varieties with all primers. Ambiguous bands that could not be clearly distinguished were not scored. The data generated from the detection of polymorphic fragments were analyzed by using Popgene32 software (Ver. 1.44) (Yeh *et al*., 2000). The polymorphism information content (PIC) was calculated by using the following equation (Botstein *et al*., 1980):

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - 2\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} p_i^2 p_j^2 = 1 - \sum_{i=1}^{n} p_i^2 - \left(\sum_{i=1}^{n} p_i^2\right)^2 + \sum_{i=1}^{n} p_i^4 \quad ,$$

Results and Discussion

Considerable genetic diversity (Iqbal *et al*., 1997; Rahman *et al*., 2002) and phylogenetic relationship (Khan *et al*., 2000) have been reported existing among cotton varieties/genotypes/strains. The 21 RAPD primers (Table 1) showed polymorphism in the 25 cotton genotypes. Each primer-template produced distinct, easily detectable bands of variable intensities.

The 19 varieties belonging to *G. hirsutum* produced more number of bands averaging 72.78 bands per variety while varieties belonging to *G. arboreum* yielded comparatively less number of bands with an average of 50.5 bands per variety. Considering all the primers and *G. hirsutum* varieties, a total of 106 bands were amplified, of which 97 (91.50%) were polymorphic, showing the mean PIC value of 0.656. The PIC values for *G. hirsutum* ranged from 0.375-0.878 (Table 1). The genetic distances ranged from 0.069-0.994 with an average of 0.371 (Table 3) indicating a relatively broader genetic base. On the other hand *G. arboreum* varieties produced a total of 94 bands, of which 84 (89.36%) bands were polymorphic with mean PIC value of 0.578. The PIC values for *G. arboreum* ranged from 0.30-0.857 (Table 1).

The genetic distances ranged from 0.27-0.50 with an average of 0.371(Table 3) again indicating a relatively broader genetic base. With RAPD primers, maximum similarity was observed between FH-87 and CIM-240 while maximum dissimilarity was observed between FH-87 and FDH-300. The number of amplification products produced per primer in *G. hirsutum* varied between 2 to 13 with an average of 5.76 bands per primer. The maximum bands were produced by the primer GL J-05 while the minimum bands were produced by the primers GL J-15, GL K-3, GL B-6, GL B-16 and GL J-11 (Table 1). The number of bands per primer for *G. arboreum* ranged from 1 to 9 with an average of 4.42 bands per primer (Table 1). Haley *et al*., (1993) reported 5.4 bands per primer in beans and Malik (1995) has reported an average of 5.2 bands per primer in wheat with RAPD primers, since the RAPD markers can be used across the species to detect the polymorphism and genetic diversity therefore the results of this study are comparable with the results mentioned by the above researchers, although the work has been conducted in other crops instead of cotton.

An average of 28.73 bands per variety was produced in the varieties belonging to *G. hirsutum* by using SSR analysis while the varieties belonging to *G. arboreum* yielded comparatively low average of 20.33 bands per variety.

A total of 52 bands were amplified by the SSR primers in 19 *G. hirsutum* varieties, of which 46 (88.46%) were polymorphic (Table 3), showing the mean PIC value of 0.290 with a range of 0.0-0.741 in *G. hirsutum* (Table 2). The genetic distances ranged from 0.075-0.691 with an average of 0.32 (Table 3). While on the other hand a total of 39 bands were produced in *G. arboreum* varieties, of which 33 (84.60%) bands were polymorphic (Table 3) with PIC value of 0.183. The PIC values for *G. arboreum* ranged from 0.0-0.744 (Table 2), their genetic distances ranged from 0.25-0.65 with an average of 0.41 (Table 3) indicating a relatively narrow genetic base.

With SSR primers, maximum similarity was observed between the varieties MNH-554 and NIAB-78 while maximum divergence was observed between MNH-149 and FDH-306. The number of amplification products produced per primer for *G. hirsutum* varied from 1 to 7 with an average of 2.52 bands per primer. The maximum numbers of bands were produced by the primer JESPR-247 while the minimum number of bands was produced by the primer pairs JESPR-281, JESPR-282, JESPR-283, JESPR-286 and JESPR-288 (Table 2).The number of bands per primer for *G. arboreum* ranged from 1 to 6 with an average of 1.76 bands per primer (Table 2). Previously, 3 bands per primer have been reported in *Gossypium* spp. (Samina *et al*., 2004).

Table 1. Number of alleles, primer sequence and PIC values for RAPD loci found in 19 genotypes of *Gossypium hirsutum* and 6 genotypes of *Gossypium arboreum*.

Sr. No.	Primer name	Sequence	*Gossypium hirsutum*		*Gossypium arboreum*	
			No. of Alleles	PIC Value	No. of Alleles	PIC Value
1.	GL DecamerA-05	AGGGGTCTTG	02	0..375	02	0.330
2.	GL DecamerA-08	GTGACGTAGG	03	0.585	03	0.488
3.	GL DecamerA-09	GGGTAACGCC	05	0.719	03	0.527
4.	GL DecamerB-06	TGCTCTGCCC	02	0.375	02	0.381
5.	GL DecamerB-16	TTTGCCCGGA	02	0.375	02	0.375
6.	GL DecamerC-11	AAAGCTGCGG	06	0.744	06	0.754
7.	GL Decamer J-01	CCCGGCATAA	07	0.776	06	0.790
8.	GL Decamer J-05	CTCCATGGGG	13	0.867	05	0.690
9.	GL Decamer J-07	CCTCTCGACA	11	0.807	09	0.851
10.	GL Decamer J-10	AAGCCCGAGG	05	0.767	05	0.65
11.	GL Decamer J-11	ACTCCTGCGA	02	0.375	02	0.381
12.	GL Decamer J-14	CACCCGGATG	09	0.855	03	0.541
13.	GL Decamer J-15	TGTAGCAGGG	02	0.376	02	0.360
14.	GL Decamer J-19	GGACACCACT	05	0.681	05	0.740
15.	GL Decamer J-20	AAGCGGCCTC	10	0.878	10	0.857
16.	GL Decamer K-03	CCAGCTTAGG	02	0.375	02	0.300
17.	GL Decamer K-07	AGCGAGCAAG	11	0.867	09	0.813
18.	GL Decamer K-08	GAACACTGGG	07	0.810	04	0.600
19.	GL Decamer K-15	CTCCTGCCAA	09	0.842	07	0.790
20.	GL Decamer K-17	CCCAGCTGTG	04	0.676	03	0.584
21.	GL Decamer K-19	CACAGGCGGA	04	0.667	04	0.670
	Average		**5.76**	**0.656**	**4.42**	**0.578**

Of the 21 RAPD and 21 SSR primers pairs 14 (66.66%) RAPD and 04 (19.04%) SSR primers were highly informative in *G. hirsutum* (Table 4). In *G. arboreum* 13 (61.90%) RAPD and 2 (09.52%) SSR primers were highly informative (Table 4).Genetic diversity reflects an affluent history of selection, migration, recombination and mating system. Additionally, the nucleotide diversity across a genome provides basis for the phenotypic level variation. A primary conclusion of the present study is that RAPD showed PIC value of 0.656 and 0.578 respectively for *G. hirsutum* and *G. arboreum* while SSR showed PIC value of 0.29 for *G. hirsutum* and 0.183 for *G. arboreum*, this fluctuation in the polymorphism information content (PIC) among *G. hirsutum* and *G. arboreum* could be attributed to the nature of the genetic material under investigation. The extent of polymorphism differs considerably between species and varieties of the same species.

Genetic diversity is normally measured as the average sequence divergence between any two individuals for given loci (Wendel & Brubaker, 1993). Some of this variation in the extant of polymorphism reflects the choice of species, but major differences are observed for random genes within a single genome. The high degree of polymorphism in this study compared to other reports appears to be due to the more diverse material which belonged to two different cultivated species of cotton. Moreover, the various cultivars within a species were selected from different agro-climatic zones.

Table 2. Number of alleles, primer sequence and PIC values for SSR loci found in 19 genotypes of *Gossypium hirsutum* and 6 genotype of *Gossypium arboreum*.

S. No.	Primer name	Sequence (5' – 3')	Repeat motif	*Gossypium hirsutum*		*Gossypium arboreum*	
				No. of Alleles	PIC Value	No. of Alleles	PIC Value
1.	JESPR-247-F JESPR-247-R	GCTTCTTCCATTTTATTCAAG CAGCGGCAACCAAAAAG	(CT)15	07	0.740	04	0.540
2.	JESPR-278-F JESPR-278-R	ACCCTTAAATCATAAGAGAAC CCGTAAGTTAAGGTACAAGG	(CA)10(GA)3	02	0.282	02	0.247
3.	JESPR-279-F JESPR-279-R	GGAGTGAAAGCTAATGCCTG CGGGTCATTGGTTGTTTTTG	(CA)28	02	0.358	02	0.382
4.	JESPR-280-F JESPR-280-R	GGAGTACAAGGACCAGCAG GGATTAACTAATTAGTTTCCGC	(GT)10	06	0.708	01	0.000
5.	JESPR-281-F JESPR-281-R	TGATTGATCCTAGTTCTACG GTCTCCTTACTTCGCAAC	(TA)4(GT)12	02	0.281	01	0.000
6.	JESPR-282-F JESPR-282-R	GGAGTACAAGGACCAGCAG CATAAGCCATGGTTGTAC	(TG)10	01	0.000	01	0.000
7.	JESPR-283-F JESPR-283-R	TCATCATGCTATTCATTGAACTAA GCAGCGAGAATTATCATGG	(TA)5(TG)14	01	0.000	01	0.000
8.	JESPR-284-F JESPR-284-R	CAAGATCCATCTGCTGATTAG GTATATACAAGTATAAAGTATTGG	(CA)25(TA)5	01	0.000	01	0.000
9.	JESPR-285-F JESPR-285-R	CCCGGATATAGTACTAAGG C ATGTATGGTGTTGAGTGC	(CA)10	02	0.327	02	0.305
10.	JESPR-288-F JESPR-288-R	CAATATAAGCACGTAAC CATGTATATACAAGTATAAAG	(TA)5(GT)11	01	0.000	01	0.000

Table 2. (Cont'd.).

S. No.	Primer name	Sequence (5' – 3')	Repeat motif	*Gossypium hirsutum*		*Gossypium arboreum*	
				No. of Alleles	PIC Value	No. of Alleles	PIC Value
11.	JESPR-292-F JESPR-292-R	GCTTGCAATCTCCTACACC GAATATGTTTCATAGAATGGC	(CTT)7	02	0.377	02	0.370
12.	JESPR-295-F JESPR-295-R	GCCTCGTTTAAGCCCATAAAC GAGGGCCATAGTCACCGG	(CTT)7	02	0.272	01	0.000
13.	JESPR-296-F JESPR-296-R	GGGTGTTACATAGAGTGTATAAAATTG TGACCTCAATTTAGAAACCC	(TCA)8(CTT)3	02	0.375	02	0.382
14.	JESPR-299-F JESPR-299-R	GCCTAGGTGGAGTTCGTG CTGAACCTGCTCCTGAATC	(CAT)9	05	0.741	02	0.339
15.	JESPR-300-F JESPR-300-R	CGCATCACAAACCAAACAC CGGAAAATGATGATGATGAAGAAG	(CTT)5(CAT)6	02	0.195	01	0.000
16.	JESPR-301-F JESPR-301-R	TGAGTTCCGAATTCCTTGG CGGGCTAAGTGTTTTTCG	(CAT)8	02	0.218	01	0.000
17.	JESPR-302-F JESPR-302-R	CACTCCTAGCTTCTTGGCATC CTGCGATCTTGGCACAG	(GAT)5	02	0.246	02	0.216
18.	JESPR-303-F JESPR-303-R	CATCGGAAAACTCTGAAC GTAGCAGTACAGATGAAAGAG	(CAT)6	06	0.636	06	0.744
19.	JESPR-305-F JESPR-305-R	CGATCCATCAAAGGCGAC CCGCCTCAGCACCATTTAC	(GAT)6	02	0.143	01	0.000
20.	JESPR-306-F JESPR-306-R	CCCCTTACATTATATTGACCTGC CCATGTGAAAAGGGGATA	(CT)10(CAT)8	02	0.375	02	0.321
21.	JESPR-286-F JESPR-286-R	GGAGGACATGGGTTTGAAC GCATGCATGTAAAATGTAATGG	(CA)30	01	0.000	01	0.000
Average				**2.52**	**0.290**	**1.76**	**0.183**

Table 3. Number of polymorphic markers, average and range of pair wise genetic distance among 19 genotypes of *Gossypium hirsutum* and 6 genotypes of *Gossypium arboreum.*

Marker system	*Gossypium hirsutum*			*Gossypium arboreum*		
	Number of polymorphic markers	Average genetic distance	Range genetic distance	Number of polymorphic markers	Average genetic distance	Range genetic distance
RAPD	97(91.50 %)	0.371	0.069-0.994	84 (89.36%)	0.371	0.270-0.500
SSR	46(88.46 %)	0.320	0.075-0.691	33 (84.60%)	0.410	0.250-0.650

Table 4. Informativeness of RAPD and SSR markers among the genotypes of the *Gossypium hirsutum* and *Gossypium arboreum*.

Marker system	Ranges of PIC Value for *G. hirsutum*			Ranges of PIC Value for *G. arboreum*		
	≤0.30 Uninformative	0.30-0.59 Moderately informative	≥ 0.60 Highly informative	≤0.30 Uninformative	0.30-0.59 Moderately informative	≥ 0.60 Highly informative
RAPD	0 (0.00%)	07 (33.33%)	14 (66.66%)	0 (0.00%)	08 (38.09%)	13 (61.90%)
SSR	12 (57.14%)	05 (23.8%)	04 (19.04%)	13 (61.9%)	06 (28.57%)	02 (09.52%)

Both RAPD and SSR primers amplified polymorphic alleles among different cotton varieties of *G. hirsutum* and *G. arboreum*. The variation in SSR is supposed to be either due to slippage of DNA polymerase during replication or unequal crossing over, consequential in differences in the copy number of the core nucleotide sequences (Yu *et al.*, 1997). SSR polymorphism occurs due to structural rearrangements and presence or absence of introgressed DNA in the amplified region (Paran & Michelmore, 1993).

The contiguous regions of SSR are highly conserved both at intra and inter-specific levels in cotton (Liu *et al.*, 2000a). SSRs have been used in genetic analysis of cotton due to their highly polymorphic nature (Reddy *et al*., 2001). Deletion or insertion of a single base or even long DNA fragment in the flanking regions has been reported as a source of variation in SSRs (Buteler *et al*., 1999). However, no clear relationship has been found between length and heterozygosity. The polymorphism in RAPD is due to single base change, deletion and insertion of DNA fragments. In this study RAPD produced comparatively more number of bands because RAPDs are arbitrary in nature and can anneal anywhere in the genome while the flanking regions of SSR are highly conserved both at intra and inter-specific levels.

Varieties belonging to *G. hirsutum* produced more number of bands as compared to *G. arboreum* varieties because *G. hirsutum* is tetraploid and *G. arboreum* is diploid in nature. The analysis of the results revealed that the varieties belonging to *G. hirsutum* and *G. arboreum* showed less similarity between them, clearly an account of belonging to two distinct species having different ploidy levels (tetraploid and diploid respectively) which share only one genome (A) of the two genomes (A & D). The varieties belonging to *G. arboreum* have same origin therefore they showed greater similarity among them. The varieties belonging to *G. hirsutum* were collected from different sources and therefore showed comparatively less similarity among them.

The molecular markers used in this study have technical differences in terms of cost, speed, amount of DNA needed, technical labor, degrees of polymorphism detected, precision of genetic distance estimates and the statistical power of tests.

Initially the RAPD technology was well suited to DNA fingerprinting because it was easy and cheap, requiring less amount of DNA and less expertise but it suffers from lack of precision of genetic distance estimates and reproducibility, to a certain degree, due to mismatch annealing (Demeke *et al*., 1997).

Microsatellite (SSRs) occurs frequently in the majority of eukaryotic genomes and are extremely informative, multi allelic and reproducible (Vos *et al.,* 1995) and are suggested to be used to overcome the limitation associated with RAPD. The application of SSR techniques to plants depends on the availability of appropriate microsatellite markers.

Keeping in view all the merits and demerits of these techniques, it is concluded that SSR markers are better than RAPD markers, if the primers are carefully screened for polymorphism, because SSR is a cost effective, informative, reliable and reproducible over repeated runs, which is very hard to achieve through RAPD technique

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(Received for publication 15 January 2010)